

# Increased motility of mesenchymal stem cells is correlated with inhibition of stimulated peripheral blood mononuclear cells *in vitro*

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## Abstract

Immunomodulatory properties of mesenchymal stem cells (MSC) are key components of their successful applications in clinical setting. However, treatments based on MSC immunomodulation need understanding of cell characteristics before cell transplantation. We used live-imaging to test the suitability of the MSC motility as a parameter for quick prediction of the immunomodulatory potential of human MSC in regulating the activity of stimulated peripheral blood mononuclear cells (PBMC) *in vitro*. Bone marrow MSC, from various donors and *in vitro* passages, were cultured with or without stimulated PBMC. After seven days, immunomodulation was assessed by measuring PBMC proliferation, IgG production and cytokine secretion in MSC and PBMC monocultures and co-cultures, and results were correlated to MSC motility. In co-culture, we observed that MSC successfully inhibited PBMC activity, reducing PBMC proliferation and IgG production compared to PBMC monoculture. MSC modulated PBMC to reduce the secretion of TNF $\alpha$  and IL-10, increase IL-6, G-CSF and MCP-1, while GM-CSF was not affected. By live-imaging tracking of cell trajectories, we observed that fast moving MSC were inhibiting more efficiently stimulated PBMC compared to slow ones. In co-culture, fast MSC were more effective in inhibiting IgG production (~30% less IgG), and secreted higher levels of IL-10 (~10% increase) and GM-CSF (~20% increase) compared to slower cells. Furthermore, fast MSC in monocultures produced 2.3-fold more IL-6, 1.5-fold MCP-1 and 1.2-fold G-CSF in comparison to slower cells. In conclusion, live-imaging cell tracking allowed us to develop an indicative assay of the immune-regulatory potential of MSC prior to *in vivo* administration.

**Key Words:** Human mesenchymal stem cells, Immunomodulatory potential, In vitro cell motility, Stem cell transplantation

## Introduction

Human mesenchymal stem cells (MSC) are multipotent stromal cells with the ability to undergo extensive proliferation<sup>[1,2]</sup>, produce a large number of cytokines<sup>[3]</sup>, and the potential to differentiate into several cell lineages, such as adipocytes, chondrocytes, myocytes and osteoblasts<sup>[4]</sup>. Because of these defining characteristics, MSC emerged as one of the favorite candidates for cell-based therapies and tissue engineering approaches. Clinical trials have shown the beneficial effects of MSC application in critical health conditions like myocardial infarction<sup>[5]</sup>, spinal cord injuries<sup>[6]</sup> and liver cirrhosis<sup>[7]</sup>. Additionally to their tissue restorative capacity, MSC also showed extensive immunomodulatory effects<sup>[8]</sup> making them potentially applicable in the treatment of immune-related diseases, such as graft versus host disease<sup>[9]</sup>, systemic lupus erythematosus and multiple sclerosis<sup>[10]</sup>.

The exact mechanism by which MSC regulate the immune system is still a matter of debate, but it is likely to be a co-effect of direct contact between cells and secretion of soluble factors. The complexity of the immunomodulatory mechanism is represented by the involvement of more than a dozen soluble factors which were shown to act on both innate and adaptive immune cells<sup>[11,12]</sup>. Among the most discussed secreted immunosuppressive factors are indoleamine-pyrrole 2,3-dioxygenase<sup>[13]</sup>, prostaglandin E2<sup>[14]</sup>, transforming growth factor- $\beta$ <sup>[15]</sup> and nitric oxide<sup>[16]</sup>. Secretion of those soluble factors is dependent on the microenvironment

of the MSC, for example tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 1- $\beta$  (IL1- $\beta$ ) or interferon- $\gamma$  (IFN- $\gamma$ ) are required for the activation of MSC induced immunomodulation<sup>[17,18]</sup>. MSC were reported to mediate a shift from a Th1 driven response to rather an anti-inflammatory Th2 response<sup>[19]</sup> which is characterized by lower levels of interleukin-2 (IL-2), IFN- $\gamma$  and TNF- $\alpha$  and higher concentrations of interleukin-4 (IL-4), IL-5, IL-10 and IL-13. MSC can also secrete interleukin-6 (IL-6) which was shown to inhibit the differentiation of monocytes into antigen presenting dendritic cells<sup>[20]</sup>. Furthermore, IL-6 produced by MSC promotes neutrophil survival and expansion when combined with granulocyte macrophage colony-stimulating factor (GM-CSF)<sup>[21]</sup>. Additionally, it was found that by producing monocyte chemoattractant protein-1 (MCP-1 or CCL2), MSC are able to inhibit IgG production of plasma cells<sup>[22,23]</sup> and induce T-cells apoptosis via the FAS ligand-dependent FAS pathway<sup>[24]</sup>.

Despite all promising researches, clinical trials with autologous MSC transplantations can have uncertain outcomes, partially caused by the wide intra- and inter-donor variability of MSC populations<sup>[25]</sup>. Such variations include diverse differentiation potential, cell morphology<sup>[26]</sup> or cell motility. Nowadays, there is a consensus that MSC therapies would be better predictable and more reproducible if we could evaluate and then use only cells with standardized characteristics.

We have demonstrated previously that *in vitro* MSC motility can be

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a useful parameter to quickly characterize and distinguish the differentiation potential of MSC populations<sup>[27]</sup>. Despite the observation that MSC were moving randomly and without directionality, we could determine that slow moving MSC were more likely to be senescent when compared with fast moving ones. However, average moving cells outperformed the rest with their ability to differentiate showing that MSC motility is a good predictor for the differentiation potential. We then wanted to investigate if a correlation with the immunomodulatory capacity of MSC also exists.

In this study, we tested the suitability of the MSC motility as a parameter for quick prediction of the immunomodulatory potential of the cells. We investigated by live-imaging microscopy the *in vitro* motility of human MSC isolated from different donors at various culture passages and correlated the velocity to their immunomodulatory capacities in co-culture with allogeneic peripheral blood mononuclear cells (PBMC). Immunomodulation was assessed by measuring the PBMC proliferation, IgG production and cytokine secretion (listed in Table 1) in the MSC and PBMC co-cultures and results were compared to PBMC and MSC monocultures.

**Table 1.** List of analysed cytokines and their expression by MSC

Cell type	Immunomodulation	Cytokines [Ref]
MSC	Pro-Inflammatory	IL-6 <sup>[3, 33]</sup>
	Anti-Inflammatory	IL-6 <sup>[3, 33]</sup> , MCP-1 <sup>[20]</sup>
PBMC	Pro-Inflammatory	TNF- $\alpha$ <sup>[38]</sup> , IL-10 <sup>[32]</sup>
	Anti-Inflammatory	IL-6 <sup>[33]</sup> , IL-10 <sup>[39]</sup> , MCP-1 <sup>[20]</sup> , G-CSF <sup>[34, 40]</sup>

## Materials and Methods

### Mesenchymal stem cells (MSC) isolation and expansion

Bone marrow (BM) samples were harvested from the iliac crest of donors undergoing spine surgery. The study was ethically approved by the ethics committee of Canton of Lucerne (Study number: 730). Written informed consent was obtained from all the participants and this procedure was also approved by the approving body. MSC were isolated from BM of ten donors (4 females and 6 males; average age: 43 years; range: 17-59 years). The BM aspirates were immediately resuspended in 3.8% sodium citrate and phosphate buffered saline (PBS, Applichem - Axonlab, Baden, Switzerland) and then filtered through a 100  $\mu$ m cell strainer to remove clots (Falcon - Faust, Schaffhausen, Switzerland). Mononuclear cells were separated by H-Lympholyte Cell Separation Media gradient centrifugation (density 1.077 g/mL; Cedarlane - Bio Concept, Allschwil, Switzerland) in Leucosep tubes (Huberlab, Reinach, Switzerland) at 800 g for 15 minutes, washed with PBS, centrifuged again at 210 g for 10 minutes and plated at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> in tissue culture flasks (TPP - Faust) in  $\alpha$ -MEM, supplemented with 10% fetal bovine serum (FBS) (both Amimed - Bio Concept), (100 units/mL) penicillin / (100 mg/mL) streptomycin, 2.5  $\mu$ g/mL amphotericin B (both Gibco - LuBioScience GmbH, Lucerne, Switzerland) at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. After two days, non-adherent cells were discarded, whereas adherent cells were cultured in growing medium consisting of DMEM/Ham's F12, supplemented with 10% foetal bovine serum (FBS) (both Amimed), (100 units/mL) penicillin / (100 mg/mL) streptomycin (Pen-Strep), 2.5  $\mu$ g/mL Amphotericin B (all Gibco) and 5 ng/mL recombinant basic fibroblast growth factor

(bFGF, Peprotech - LuBioScience), followed by media change three times per week. At 80% confluency, MSC were frozen and stored at -150°C.

### Phenotypic characterization of MSC

Bone marrow-derived mononuclear cells ( $1 \times 10^6$  cells) were plated in 10 cm-Primaria cell-culture dishes (Falcon) and cultured with growing medium. After 14 days, cell colonies were washed with PBS, fixed with 100% methanol, and stained with Giemsa solution (all these reagents were from Applichem).

Following cell culture expansion, CD44, CD90 and CD105 positive and CD14 negative MSC markers were analyzed by flow cytometry. Briefly, cells were incubated with CD14-FITC (NB100-77759, Novus Biological - LuBioScience), CD44-FITC (NBP1-41278, Novus Biological), CD90-FITC (NBP1-96125, Novus Biological) and CD105-FITC (MCA1557A488T, AbD Serotec - LuBioScience) antibodies in PBS for one hour at 4°C, washed and resuspended in PBS. Cell fluorescence was evaluated with FACScalibur flow cytometer (Becton Dickinson) and data were analyzed using FlowJo v.10.0 software (Treestar, Ashland, OR, USA).

### Peripheral blood mononuclear cells (PBMC) isolation and characterization

Venous blood (45 ml) was collected from a healthy male donor (27 years old) in S-Monovettes containing EDTA (S-Monovettes, 9 ml K3E; Sarstedt, Nümbrecht, Germany). Peripheral blood mononuclear cells (PBMC) were isolated by H-Lympholyte Cell Separation Media gradient centrifugation in a Leucosep tube at 800 g and room temperature, for 20 minutes. The PBMC-containing buffy coat was carefully retrieved and washed with PBS, followed by a centrifugation at 210 g and room temperature for 10 minutes. The obtained PBMC pellet was resuspended in RPMI-1640 medium (Amimed; Bio Concept) supplemented with 10% FBS, Pen-Strep and amphotericin B.

Flow cytometric analysis of PBMC with CD4 (APC Mouse Anti-Human CD4 Clone RPA-T4, BD Bioscience, Allschwil, Switzerland) and CD8 (PE Mouse Anti-Human CD8 Clone RPA-T8, BD Bioscience, Allschwil, Switzerland) surface markers was performed after seven days in co-culture with MSC and in PBMC monoculture. Cells were incubated with antibodies in PBS for 15 minutes at room temperature, washed and resuspended in PBS. Cell fluorescence was evaluated with FACScalibur flow cytometer and data were analyzed using FlowJo v.10.0 software.

### PBMC stimulation before co-culture

PBMC were stimulated in a cocktail adapted from a previous work<sup>[28]</sup>, consisting of 60 ng/mL human recombinant interleukin-2 (IL-2), 25 ng/mL Interleukin-10 (IL-10), 100 ng/mL Interleukin-21 (IL-21) (all from BioBasic Inc.; Stephan Klee Trading and Consulting, Sissach, Switzerland), the synthetic unmethylated oligodeoxynucleotide deoxycytosine-deoxyguanosine (CpG2429: tcgtcgttttcggcgccgcgcg, 360 nM<sup>[29]</sup> Microsynth AG, Balgach, Switzerland) and 2.5  $\mu$ g/mL pokeweed mitogen (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in RPMI-1640 medium (Amimed; Bio Concept) + 10% FBS. After addition of the stimulating cocktail, PBMC were incubated overnight at 37°C in the incubator (5% CO<sub>2</sub>), then washed with PBS, centrifuged at 210 g at room temperature for 10 minutes, and re-suspended in media for co-culture with MSC, or as a control.

### MSC and PBMC co-cultures

MSC were split in three groups for co-culture with PBMC, *in vitro* cell tracking and cell metabolic activity (day 0). To prepare co-

culture with PBMC, *in vitro* cell tracking and cell metabolic activity (day 0). To prepare co-culture with PBMC, MSC ( $2 \times 10^4$  cells/well) were seeded in 96-well plates (each donor in quadruplicate; flat bottom culture test plates; TPP) in growing medium, and left for plastic attachment for 4 hours. After the incubation time, growing medium was removed and stimulated and unstimulated (negative control) PBMC ( $1.5 \times 10^5$  cells/well) were co-cultured with MSC in a final volume of 300  $\mu$ l/well of RPMI-1640 medium (Amimed; Bio Concept) supplemented with 10% FBS, (100 units/mL), Pen-Strep and amphotericin B. This co-culture was maintained for 7 days at 37°C and 5% CO<sub>2</sub>.

The ratio MSC:PBMC used (2:15) lies in range of previous work on immunosuppression<sup>[30]</sup>. To study the correlation between MSC immunomodulatory capacity and cell motility, our preliminary test showed that MSC inhibited more efficiently IgG production at the ratio of 2:15 than 1:15.

### Cell metabolic activity

MSC ( $2 \times 10^4$  cells/well) were seeded in 96-well plates (each donor in triplicate; flat bottom culture test plates) in growing medium, left overnight to adhere to the plate and then the cell activity was assessed by resazurin reduction assay. MSC were incubated (day 1) in resazurin solution - 15 ng/mL resazurin, 2.5 ng/mL methylene blue, 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide (all Applichem) in growing medium without bFGF - for 6 hours and bottom well fluoresce absorbance was measured ( $\lambda_{ex}$  = 535 nm and  $\lambda_{em}$  = 595 nm) using a Multimode Detector (DTX 880; Beckman Coulter, Nyon, Switzerland).

### In vitro cell tracking of MSC

MSC (day 0) at *in vitro* passage 2-3 (n=7) and passage 6 (n=3) were plated at a density of  $5.6 \times 10^3$  cells/cm<sup>2</sup>. After 3 hours, the movements of the adherent cells started to be recorded using an inverted phase-contrast microscope equipped with a high-sensitive camera (Olympus, Tokyo, Japan, <http://www.olympus-global.com>) at 40X magnification. The interval between image acquisitions was 10 minutes, using the Xcellence software program (Olympus) over a 24-hour period. Video sequences were analyzed using ImageJ (NIH, Bethesda, MD, <http://www.nih.gov/ij>) and the plugin MTrackJ, which allows manual tracking of individual cell trails. Analyses were only made for cells moving within the plane of focus. The full length of the track was determined as the distance from the first point to the last point of the track, and the cell speed was measured as mm/day. We defined three representative groups for cell motility: slow moving MSC (0.1-0.2 mm/day), medium moving MSC (0.4-0.5 mm/day) and fast moving MSC (0.9-1.0 mm/day).

### PBMC proliferation assay

Before stimulation, the isolated PBMC were stained with the fluorescent cell proliferation indicator CytoTell green (AAT Bioquest; LuBioScience) according to the manufactures protocol (20 minutes incubation at room temperature in 1:600 diluted dye). The proliferative response is the percentage of cells that underwent at least 1 cell division. The proliferative response of stimulated PBMC in co-cultures (n=10) and PBMC controls (n=1) was measured after 7 days of incubation by flow cytometry (total of 30'000 events gated) using the intracellular CytoTell dilution method and the viability staining solution 7-AAD (eBioscience, Vienna, Austria). Data was analyzed using FlowJo software.

### IgG production assay

After 7 days of incubation, the amount of secreted IgG antibody in the supernatant of stimulated co-cultures (n=10), PBMC controls

(n=1) and reference donors (n=42; age range: 23-58 years (mean: 42.8 years), and interquartile range IQR: 19) was quantified using a standard indirect ELISA, as follow: a 96-well plate (Nunc MaxiSorp, Fluka Chemie GmbH, Buchs, Switzerland) was coated with 1 ng/ $\mu$ L Protein A (LuBioScience) in PBS and incubated overnight at 4 °C, then washed with PBS with a plate washer (Beckman-Coulter, Nyon, Switzerland), and non-specific binding sites were blocked for 2 hours at room temperature with blocking solution containing 5% TopBlock (LuBioScience) in PBS. After washing wells with PBS, cell culture supernatants (in triplicate for each donor) – cleared of cell debris by centrifugation and diluted threefold in PBS – were incubated for 2 hours at room temperature. Supernatants were then removed and the wells washed with PBS. Anti-human-IgG HRP-conjugated secondary antibody (A80-119P Bethyl) diluted 1:5000 in blocking buffer was incubated for 1 hour at room temperature. IgG were determined by colorimetric measurement of the product of the enzymatic reaction mediated by HRP and o-phenylenediamine solution (15.3 mg/mL in citrate buffer, pH 5.0, Applichem – Axonlab). The reaction was stopped with 10% sulphuric acid. Absorbance was measured at 450 nm by DTX 880 Multimode Detector and IgG concentration (ng/mL) was determined from dilutions of purified human IgG (Bethyl – LuBioScience).

### Cytokine measurements

The concentrations of cytokines and chemokines were analyzed in the supernatants of monoculture of MSC, and co-cultures of PBMC and MSC after 7 days incubation, by Bio-Plex Pro Cytokine, Chemokine and Growth Factor Assay (Bio-Rad Laboratories AG – Cressier, Switzerland). Data were collected and analyzed using a Bio-Rad Bio-Plex 200 instrument equipped with Bio-Plex Manager software (Bio-Rad). We measured the concentrations of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP1) and tumor necrosis factor-alpha (TNF- $\alpha$ ).

### Statistical analysis

Cytokine and IgG concentrations, and proliferative responses were presented in bar charts as mean  $\pm$  standard deviation (SD). Non-parametric Mann-Whitney-Wilcoxon U-test for related variables was used to determine significant differences between samples, as a normal distribution of the data could not be guaranteed in this data set. For all tests,  $p < 0.05$  was considered significant. Data analysis was performed with SPSS 24.0 for Windows (SPSS Inc.).

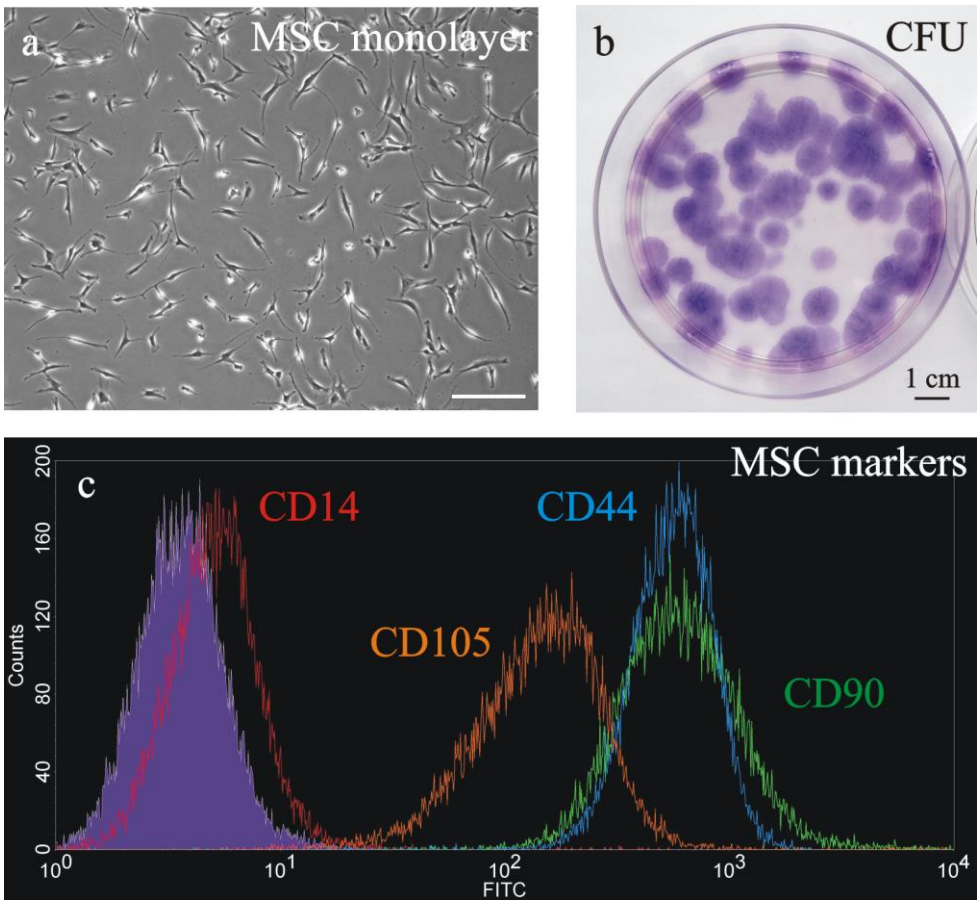
Scatterplot were produced for culture concentration versus cell motility and smoothed via semiparametric regression models using the mixed model representation of penalized splines<sup>[31]</sup> as implemented in the SemiPar package in R (R Foundation for Statistical Computing, Vienna, Austria, <http://www.r-project.org>). Scatterplots and scatterplot smoothers were computed using R, version 2.14.2, for Windows (R Foundation for Statistical Computing). The confidence bands represent the certainty about the average trend. They capture the high variation in the data points. A single future prediction may not be well accordant to the suggested trend as interpolated; however, on average (of many predictions) it will be.

## Results

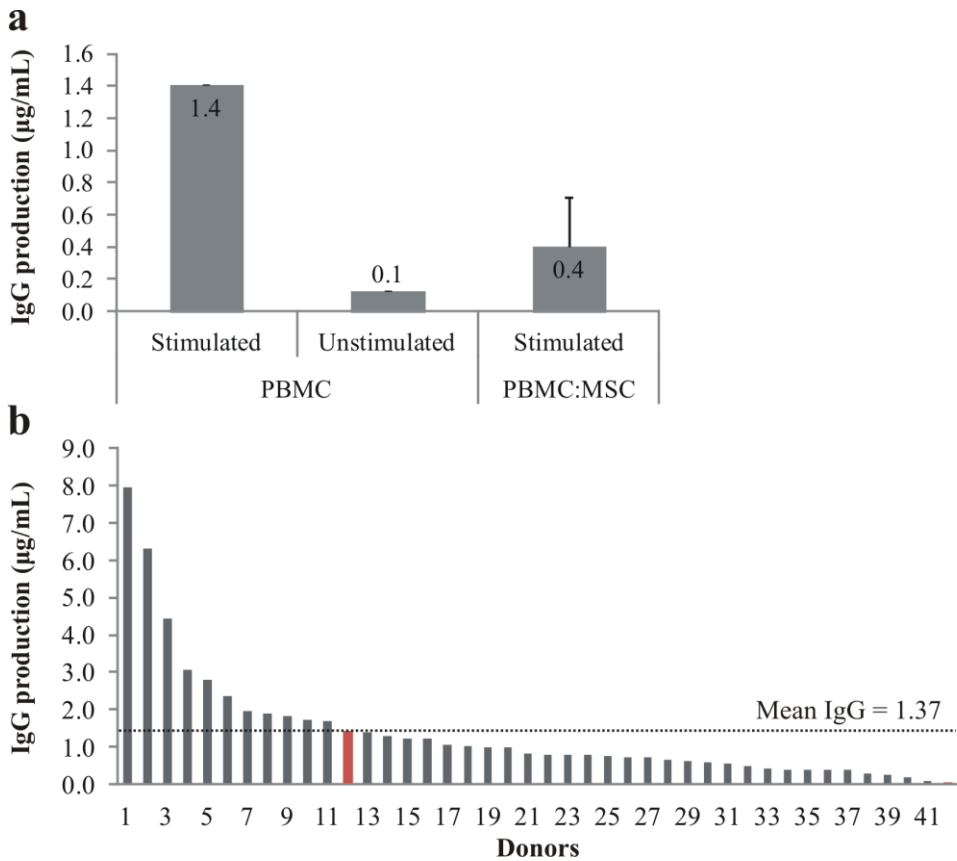
### Mesenchymal stem cells (MSC) characterization

The bone marrow-isolated MSC were initially characterized by their elongated fibroblastic cellular phenotype (Figure 1A) and by their ability to generate colony forming units (Figure 1B). Furthermore,





**Figure 1** MSC characterization was based on their peculiar fibroblast-like morphology (a, entire scale bar is 200  $\mu$ m), ability to form colonies (stained with Giemsa; b) and expression of the positive markers CD44 (blue line), CD90 (green line), CD105 (orange line) and the negative marker CD14 (red line), tested by flow cytometry analysis (grey line = unstained control; c).



**Figure 2** Effects of stimulation cocktail on IgG production by PBMC. After seven days culture, the immuno-suppressive action of MSC on PBMC (a) was determined by measuring IgG production in stimulated (n=1) and unstimulated (n=1) PBMC monocultures, as well as in stimulated PBMC and MSC co-cultures (n=10; data represented as mean  $\pm$  SD). On the bottom (b), comparison of IgG production between 42 donors (red bar = sample used to test the immuno-suppressive action of MSC).

MSC were characterized by flow cytometry analysis with the positive mesenchymal stem cell markers CD44, CD90, and CD105 and the negative monocyte-related marker CD14 (Figure 1C).

#### *IgG production by peripheral blood mononuclear cells (PBMC) in co-culture*

IgG production inhibition by MSC was tested on stimulated allogeneic PBMC in co-culture for 1 week (Figure 2A). Monocultures of MSC and PBMC (unstimulated or stimulated) served as a control. When co-cultured with MSC, stimulated PBMC decreased IgG production by 71%, while unstimulated PBMC IgG production was only a tenth of the stimulated control. Overall, PBMC production of IgG by the donor used in this project was very close (1.41 µg/ml) to the mean IgG production calculated out of 42 donors (1.37 µg/ml, Figure 2B).

#### *MSC modulated peripheral blood mononuclear cells (PBMC) activity in co-culture*

After seven days culture, stimulated (column A) and unstimulated (column B) PBMC, and stimulated PBMC isolated from co-culture with MSC (column C) characterization was based on gating light scattered by cells (Figure 3: top row), CD4/CD8 ratio of T-cells (Figure 3: middle row) and cell morphology (Figure 3D). The viability (7-AAD assay) of the stimulated PBMC in monocultures was 95% and in co-cultures 96%. Forward (FSC) and side scattering profiles of stimulated PBMC showed a large spreading and activation of cells compared to unstimulated PBMC, and the results in monoculture were similar to co-culture with MSC. Analysis of mean FSC intensity showed that stimulated PBMC increased cell size of 26% in comparison to unstimulated PBMC, and in co-culture with MSC only of 13%. CD4/CD8 ratios of unstimulated PBMC in monocultures and in co-cultures with MSC were identical (ratio = 1.55), while stimulated PBMC in monoculture (ratio = 0.70) had a lower ratio compared to PBMC in co-culture (ratio = 1.09). Immunosuppression by MSC significantly prevented blastogenesis of stimulated PBMC already after three days of co-culture.

The immunosuppressive effects of MSC on PBMC were determined by cell proliferative response of CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells (Figure 4: top rows). MSC inhibited the proliferation of both CD4<sup>+</sup> (Figure 4D) and CD8<sup>+</sup> T-cells (Figure 4E), especially CD4<sup>+</sup> T-cells (2.6-fold) in comparison to CD8<sup>+</sup> T-cells (1.6-fold), while unstimulated PBMC were not dividing at all.

We analyzed the concentration of eight inflammation-related cytokines in supernatants of controls (monocultures) and co-cultures (Figure 5), namely interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1), interleukin-10 (IL-10), tumor necrosis factor (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF). The concentrations of interleukin-2 and -4 (IL-2 and IL-4) were at or below the detection limit (data not shown). Co-culture of MSC with stimulated PBMC led to substantially increased IL-6 (Figure 5A), G-CSF (Figure 5B) and MCP-1 (Figure 5C) concentrations. In comparison to stimulated PBMC monoculture, the average cytokine increase corresponded to 340-fold for IL-6, 600-fold for G-CSF and 30-fold for MCP-1. Furthermore, the increase in G-CSF significantly correlated with *in vitro* culture passage of MSC. In stimulated PBMC:MSC co-culture, late passage MSC (P6) produced ~20% more G-CSF ( $p < 0.01$ ) and ~10% more IL-6 ( $p < 0.05$ ) in comparison to early passage MSC (P2-P3) (data not shown). On the other hand, the presence of MSC inhibited the production of TNF-α (Figure 5D) and IL-10 (Figure 5E), 3.5- and 2.5-fold respectively. GM-CSF concentrations were not influenced by MSC (Figure 5F).

MSC modulated the secretion of cytokines also in unstimulated PBMC co-culture, resulting in a 400-fold increase of IL-6, 3-fold increase of G-CSF and 5-fold increase of MCP-1 productions, compared to PBMC monoculture. Although IL-10 and TNF-α levels were unaffected by the MSC, GM-CSF was inhibited 1.8-fold in comparison to PBMC monoculture.

Monoculture of MSC produced mostly IL-6 (2.7 ng/ml) followed by MCP-1 (2.3 ng/ml), and in lower amount GM-CSF (140 pg/ml), G-CSF (50 pg/ml), TNF-α (23 pg/ml) and IL-10 (8.4 pg/ml). Besides, compared to unstimulated cells, stimulation of PBMC in monoculture increased the production of TNF-α, IL-10 and GM-CSF, and reduced MCP-1 (no changes in IL-6 and G-CSF levels).

#### *MSC in vitro motility and immunosuppressive potential*

We measured cell motility of MSC by time-lapse microscopy recording. For each donor, 70 MSC were tracked for 24 hours and their motility was plotted against proliferative response of CD4<sup>+</sup> T-cells (Figure 6A) and CD8<sup>+</sup> T-cells (Figure 6B), and IgG production (Figure 6C) by stimulated PBMC in co-culture. We observed that mostly slow and fast moving MSC suppressed PBMC proliferation and IgG production, even though changes in proliferation were small in both cases. Slow moving MSC (0.1-0.2 mm/day) corresponded to CD4<sup>+</sup> T-cells proliferative response of 11.6%, medium moving MSC (0.4-0.5 mm/day) of 12.2% and fast moving MSC (0.9-1.0 mm/day) of 11.9%. Furthermore, slow moving MSC corresponded to CD8<sup>+</sup> T-cells proliferative response of 38.4%, medium moving MSC of 40.3% and fast moving MSC of 39.3%.

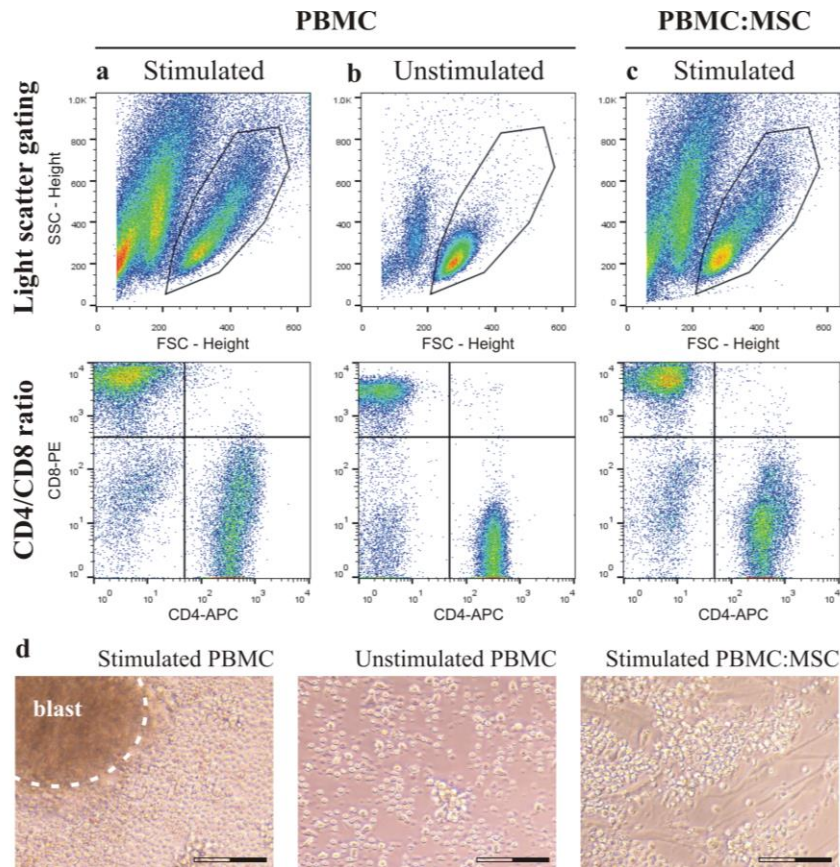
PBMC produced in correspondence of slow MSC 80% IgG (356 µg/ml), medium speed MSCs 100% (447 µg/ml) and fast moving MSCs 77% (345 µg/ml). The metabolic activity of MSC was plotted against cell motility (Figure 6D) and as expected fast moving MSC were also the most metabolically active. Compared to slow moving MSC, fast moving MSC were ~20% more active and medium moving ~10%.

#### *MSC in vitro motility in monoculture and cytokine concentrations*

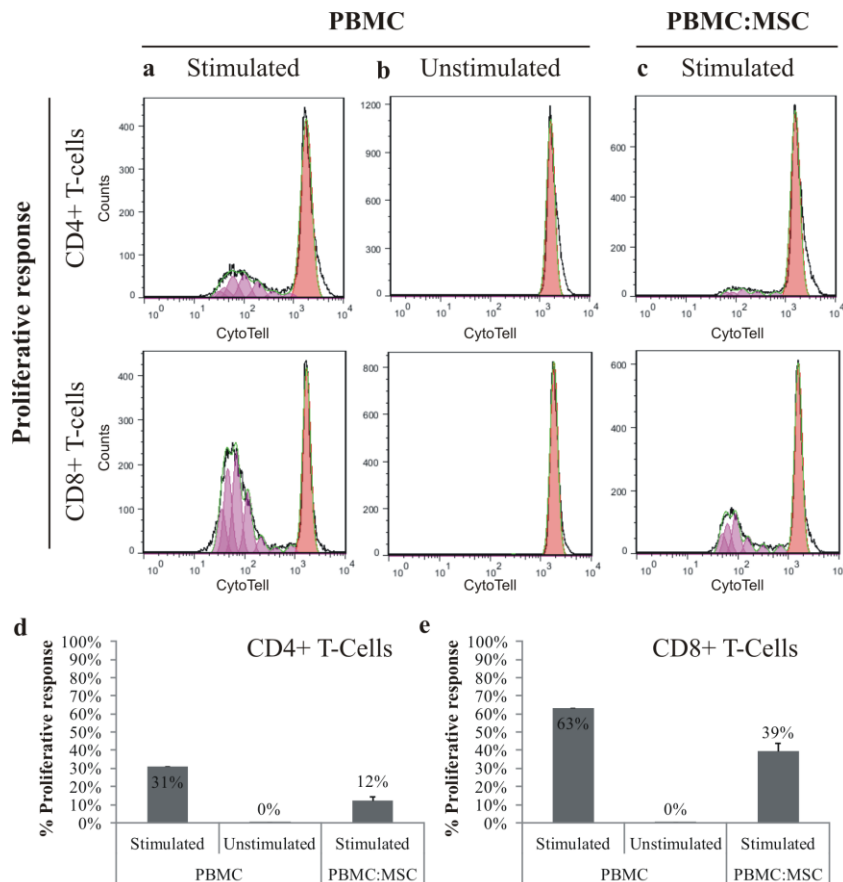
The motility of MSC was plotted against cytokine concentrations in monocultures after one week of incubation (Figure 7). Fast moving stem cells produced 2.3-fold more IL-6 (Figure 7A), 1.2-fold more G-CSF (Figure 7B) and 1.5 fold-more MCP-1 (Figure 7C) than slower speed cells. On the other hand, GM-CSF (Figure 7E) was moderately reduced (6% difference between slow and fast) with increasing MSC speed. Secretion of IL-10 (Figure 7D) and TNF-α (Figure 7F) by MSC was low and independent of MSC motility.

#### *MSC in vitro motility influence on cytokine production in co-culture with stimulated PBMC*

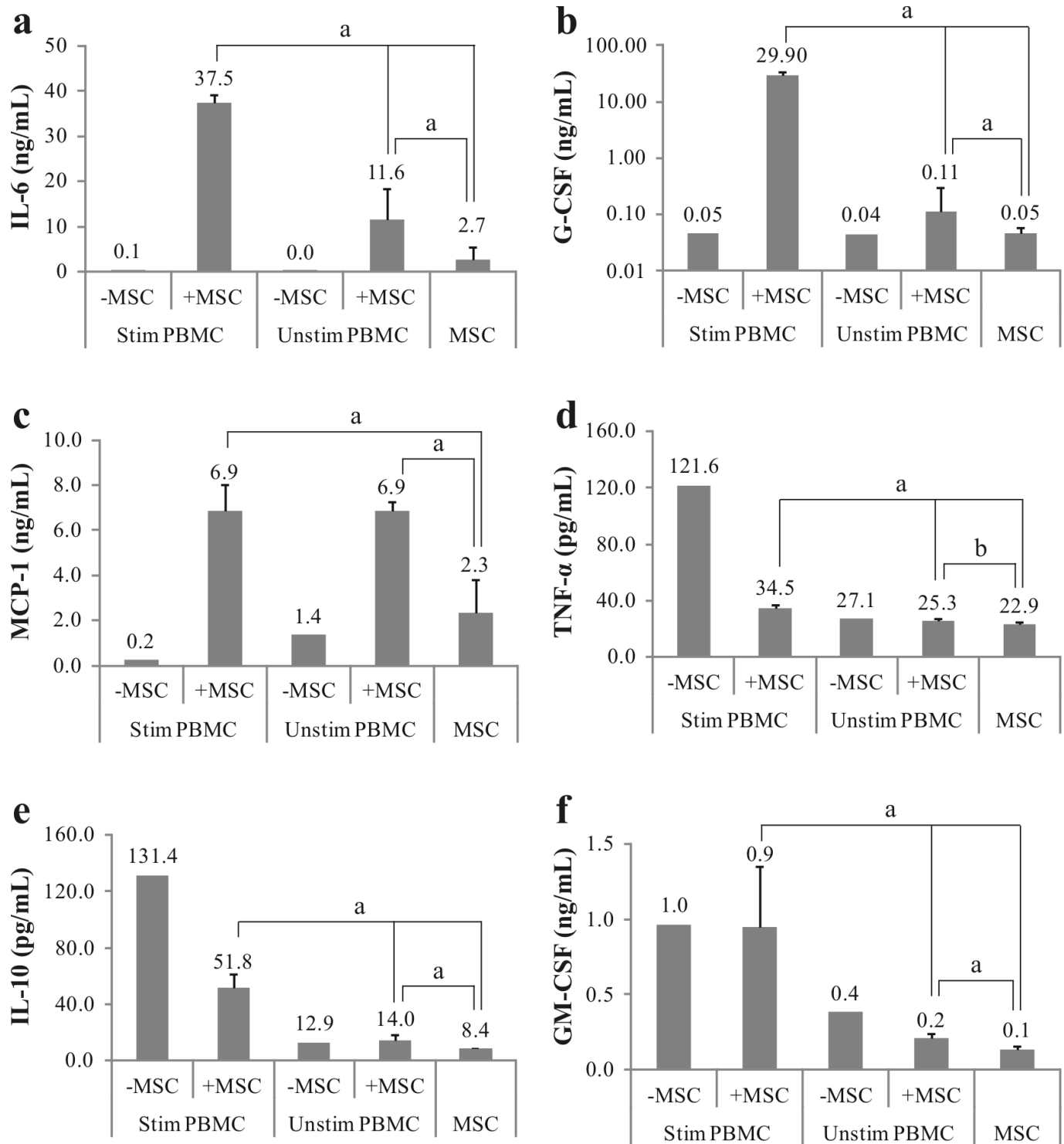
In co-culture with stimulated PBMC, cell motility of MSC was plotted against cytokine productions after one week of incubation (Figure 8). Although IL-6 concentration was strongly increased compared to MSC monoculture, the difference between slow and fast MSC was only 1.6% (Figure 8A). Also, the concentrations of G-CSF (Figure 8B) and TNF-α (Figure 8F) were independent of MSC motility with a maximum difference of 3% respectively 4% between speeds. The production of MCP-1 (Figure 8C) was negatively correlated with MSC motility. In correspondence of slow MSC, MCP-1 production was 14% higher compared to fast MSC. On the other hand, increased IL-10 (Figure 8D) and GM-CSF (Figure 8E) were produced in correspondence of fast MSC. IL-10 and GM-CSF differences between slow and fast cells were 12% and 18% respectively.



**Figure 3** After seven days culture, stimulated (column a) and unstimulated (column b) PBMC, and stimulated PBMC (column c), isolated from co-culture with MSC, were morphologically characterized. Images from a representative sample show light scattered gating of cells (side scatter, SSC, and forward scatter, FSC; top row) and CD4/CD8 ratio of T-cells (middle row). On the bottom (d), microphotographs taken after three days show the presence or lack of lymphocyte blast formation in the same samples (entire scale bar is 100  $\mu$ m).

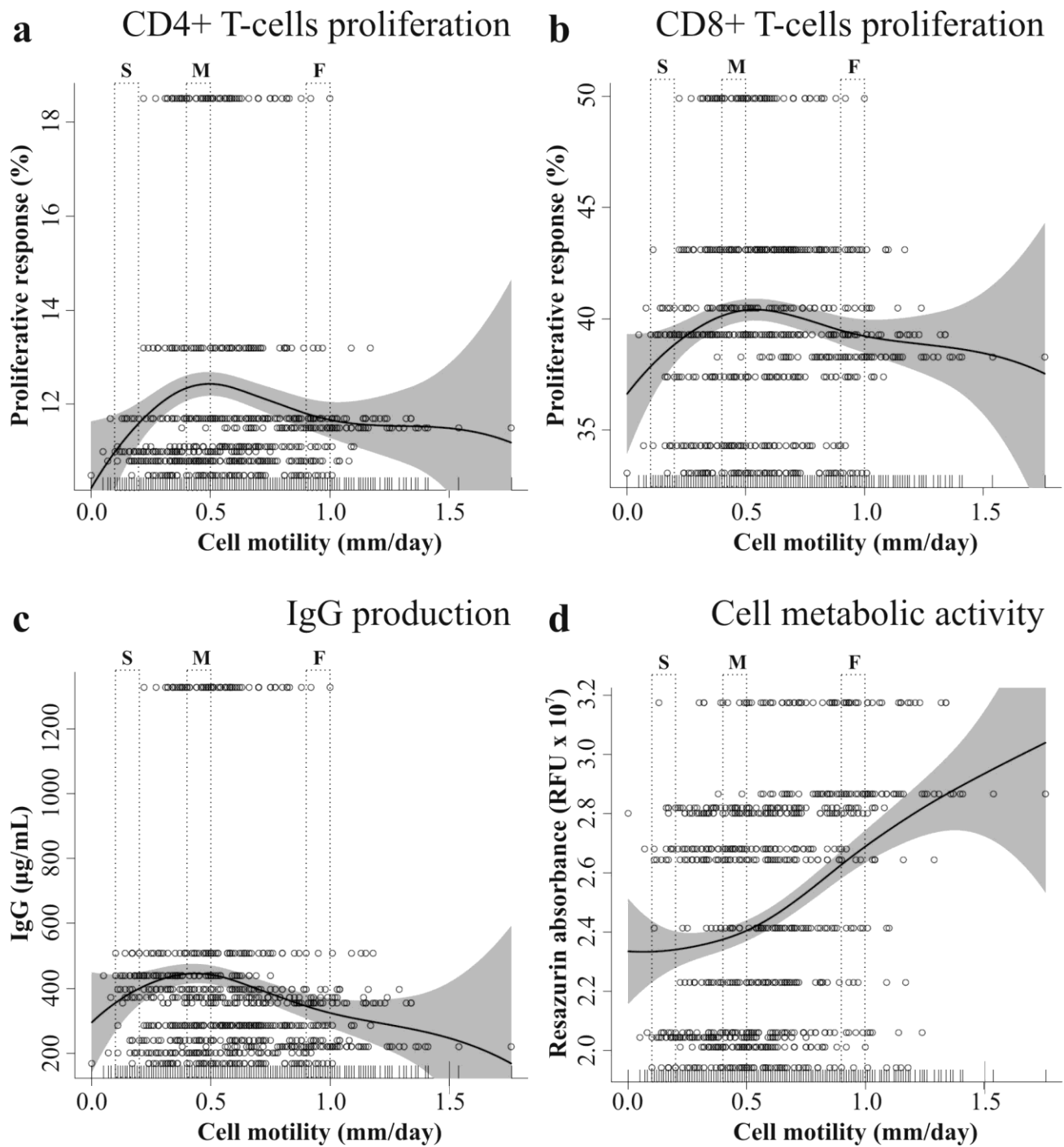


**Figure 4** After seven days culture, effectiveness of immunosuppressive action of MSC on PBMC was determined by measuring cell proliferative response of stimulated (column a) and unstimulated (column b) PBMC, and stimulated PBMC (column c), isolated from co-culture with MSC. Top rows represent the proliferative response of CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells, in a representative sample (CytoTell staining). Results of the proliferative response of CD4<sup>+</sup> T-cells (d) and CD8<sup>+</sup> T-cells (e) were summarized (stimulated (n=1) and unstimulated (n=1) PBMC monocultures, stimulated PBMC and MSC co-cultures (n=10); data represented as mean  $\pm$  SD).



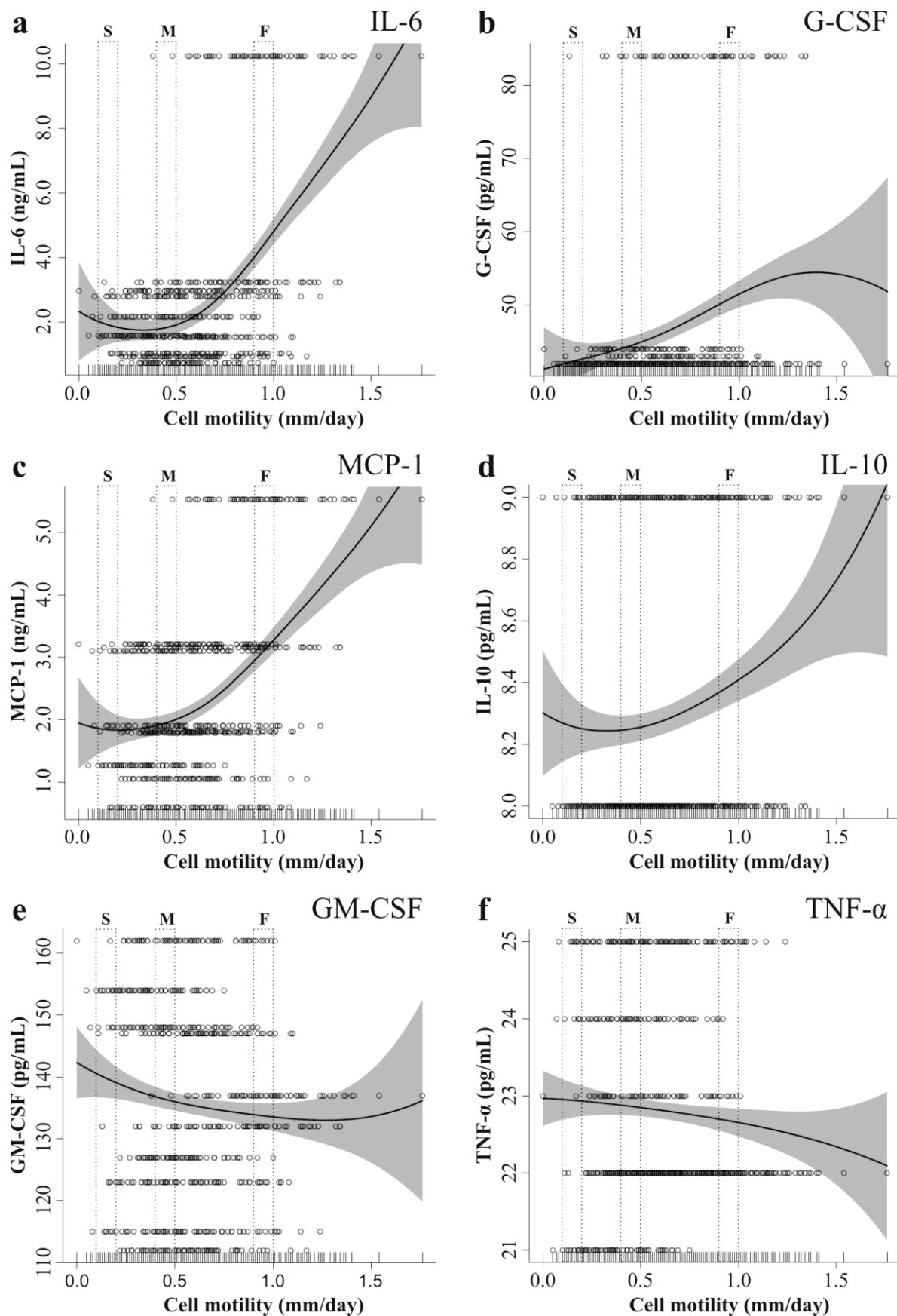
**Figure 5** MSC and PBMC had a reciprocal influence in the production of cytokines. After seven days, supernatant concentration of IL-6 (a), G-CSF (b), MCP-1 (c), TNF- $\alpha$  (d), IL-10 (e) and GM-CSF (f) was measured in MSC monoculture ( $n=10$ ), stimulated ( $n=1$ ) and unstimulated ( $n=1$ ) PBMC monocultures, and co-culture with or without MSC ( $n=10$ ). Data was acquired by Bio-Plex analysis and represented as mean  $\pm$  SD ( $a = p < 0.01$ ;  $b = p < 0.05$ ).



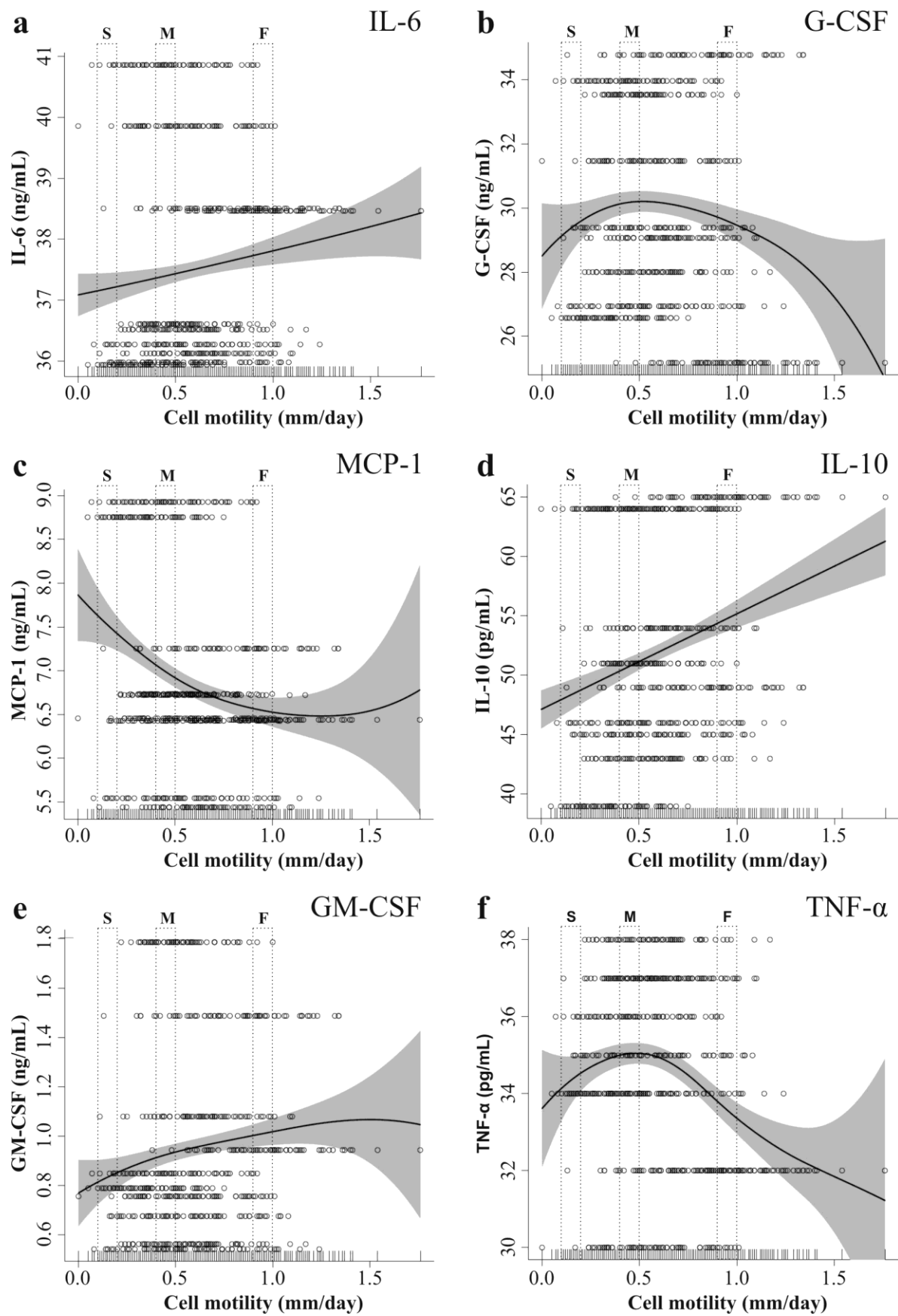


**Figure 6** Proliferative response of CD4<sup>+</sup> T-cells (a) and CD8<sup>+</sup> T-cells (b), and IgG production (c) of stimulated PBMC in co-culture with MSC plotted against cell motility. The metabolic activity (d) of MSC in monoculture was also plotted against cell motility. Each row (70 cells) represents a MSC population. Abbreviations: O = single cells; — = penalized spline smoother; grey area = confidence band. (S = slow, M = middle, F = fast moving cells)





**Figure 7** MSC monoculture concentration of IL-6 (a), G-CSF (b), MCP-1 (c), IL-10 (d), GM-CSF (e) and TNF- $\alpha$  (f) plotted against cell motility. Each row (70 cells) represents a MSC population. Abbreviations: O = single cells; — = penalized spline smoother; grey area = confidence band. (S = slow, M = middle, F = fast moving cells)



**Figure 8** Stimulated PBMC + MSC co-culture concentration of IL-6 (a), G-CSF (b), MCP-1 (c), IL-10 (d), GM-CSF (e) and TNF-α (f) plotted against cell motility. Each row (70 cells) represents a MSC population. Abbreviations: O = single cells; — = penalized spline smoother; grey area = confidence band. (S = slow, M = middle, F = fast moving cells)

## Discussion

In this study, we found that *in vitro* mesenchymal stem cells (MSC) motility correlates to some aspects of their immunosuppressive potential and production capacity of some cytokines. MSC immunomodulation effects were determined in mixed lymphocyte reaction assay, a broadly used method which provides direct contact and allows most fundamental interactions between cells. In co-cultures with peripheral blood mononuclear cells (PBMC), PBMC blastogenesis was prevented in all MSC co-cultured samples and MSC successfully inhibited CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells proliferation but also the production of IgG. We stratified the MSC populations according to our empirical observations in three representative cell motility groups: slow moving (0.1-0.2 mm/day), medium moving (0.4-0.5 mm/day) and fast moving (0.9-1.0 mm/day). In co-culture with PBMC, fast moving MSC inhibited more effectively IgG production and proliferation of PBMC in comparison to slower cells, and were the most metabolically active. Interestingly, in the case of PBMC co-cultured with fast MSC, reduced secretion of G-CSF and TNF- $\alpha$  were observed, along with increased IL-10 secretion. This particular combination of cytokine secretion is peculiar of M2 phenotype of macrophages (involved in parasite control, tissue remodelling, immune regulation and phagocytic activity), which are characterized by higher anti-inflammatory potential compared to M1 macrophages (producing pro-inflammatory cytokines, mediating resistance to pathogens and promoting Th1 responses)<sup>[32]</sup>. The ability of MSCs to shift macrophages toward an M2 phenotype *in vitro* has been already described<sup>[33]</sup>, and our data on secretion of cytokines suggest that fast moving MSC correlate better to M2 phenotype shift. Cytokine expression analysis of MSC in monoculture showed that IL-6 and MCP-1 were secreted in large amount by faster cells compared to slower ones. As a limitation, we cannot discuss properly the secretion origins of MCP-1 in co-cultures because in our experimental setting we cannot discriminate which type of cell – MSC or PBMC – produced this cytokine.

Our results suggest that within the same MSC population, sub-groups of cells have different roles in modulating the action of PBMC. There might be a possibility that the observed intra-donor variability of MSC motility extrapolates even to the stem cell variability between people. This speculation is supported by the observation that MSC isolated from patients with multiple sclerosis had functional differences compared to healthy controls, such as reduced anti-proliferative capacity and lower secretion of anti-inflammatory cytokines<sup>[34]</sup>. The dynamics occurring between sub-populations of MSC with different cell motility may play an important role for their overall immune-regulatory capacity. To better understand these dynamics, we also investigated the release of cytokines and chemokines by MSC, which are known to play a key role in the suppressive signalling.

Despite the complex nature of cytokine signalling, in co-cultures we could discriminate distinct responses related to MSC regulatory abilities. The correlation between MSC motility and immunomodulation was based on the measurement in culture medium of the cytokines IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , G-CSF, GM-CSF and MCP-1, shown previously to be relevant because of their anti- and pro-inflammatory properties and expression by both MSC and PBMC (Table 1). Stimulated PBMC secreted large amount of IL-10, TNF- $\alpha$  and GM-CSF, while unstimulated PBMC produced mostly MCP-1 and GM-CSF. On the other hand, MSC in monocultures produced only relevant amount of MCP-1 and IL-6, and negligible quantity of the other cytokines, as previously described in the literature<sup>[35]</sup>. No cross-talk from the cytokines used in the stimulation cocktail was expected since the pre-stimulated PBMC

were thoroughly washed ahead of their culturing.

The immuno-suppressive action of MSC in co-culture with stimulated PBMC was demonstrated by the reduced PBMC secretion of pro-inflammatory cytokines (TNF- $\alpha$ ) and increased MSC secretion of anti-inflammatory cytokines (IL-6, MCP-1 and G-CSF). Unexpectedly, the anti-inflammatory cytokine IL-10 was reduced by MSC, but a growing body of evidence shows that IL-10 action is multifactorial and in some settings may not be the previously thought inhibitor of immune functions<sup>[36,37]</sup>. Furthermore, IL-10 was described to stimulate IgG isotype switch in B-lymphocytes<sup>[38]</sup> and ultimately has been used as a main component of PBMC stimulation cocktail. The secretion by stimulated PBMC of another important cytokine, IL-6, was observed in co-culture with MSC, indicating an anti-inflammatory action by IL-6 in this particular setting<sup>[39,40]</sup>. Also MCP-1 had an anti-inflammatory pattern, where its expression was higher in both co-cultures compared to monocultures. Not surprisingly, the other immuno-modulatory molecule G-CSF – which has also been proven to have an anti-inflammatory role<sup>[41]</sup> – was massively upregulated when MSC were co-culture with stimulated PBMC. *In vivo*, G-CSF is a hematopoietic cytokine produced by monocytes, macrophages, fibroblasts, and endothelial cells which regulates neutrophil mobilization during infections and inflammations<sup>[42]</sup>. We speculate that such a large production of G-CSF was triggered from MSC, what might mimic the innate immune-activation – namely instant blood-mediated inflammatory and anti-inflammatory reactions – observed *in vivo* after systemic injection of MSC<sup>[43]</sup>. As a matter of evidence<sup>[44]</sup>, *in vitro* long-term expansion of MSC has been related to impaired inflammatory modulation and we observed the same pattern: co-cultures with high passage MSC (P6) were significantly producing more IL-6 and G-CSF than early passage MSC (P2 and P3). Similar results were reflected in a phase II clinical trial for the treatment of acute graft versus host disease (GvHD) where MSC transplantation failed to prevent the development of grade II acute GvHD in one patient because increased gene expression of IL-6 and CSF which possibly may have promoted T- and B-cell proliferation and macrophages activation<sup>[45]</sup>. Among all cytokines analysed, GM-CSF concentration was not affected by the presence of MSC and we concluded that it might not be involved in the immunomodulation process.

The limitation of our study is the experimental application of a single PBMC donor. However, the aim of this study was to compare MSC behaviours without introducing further confounders. Indeed, the results presented in this work focus on MSC cell motility and immunomodulatory potential rather than the effects of multiple sources of PBMC. To avoid misleading results, the choice of the PBMC donor was based on IgG production and selecting the closest donor to the mean value of the investigated population. Further limitation, is that we did not examine neither the functional outcomes that cell motility might have on specific immune cell subsets, nor the correlation of MSC hypoimmunogenicity to *in vitro* cell tracking.

## Conclusion

We showed that MSC motility is a prospective indicator for MSC immunomodulatory potential. Although the heterogeneity of cell motility in MSC populations reduce the accuracy of the proposed method, it can be fruitfully used to indicate the overall potential of cell populations, especially in those with lower variability. In monoculture, fast MSC were more metabolically active and produced larger amounts of G-CSF, IL-6 and MCP-1. In co-culture with stimulated PBMC, fast MSC inhibited more efficiently IgG production, and correlated with higher IL-10 concentrations. The

process of standardization of cell production – *i.e.* MSC extraction, expansion and administration – is fundamental in the application of MSC as a treatment. We believe that *in vitro* cell tracking can contribute to a better understanding of the immunomodulatory properties of MSC, beyond the standard MSC molecular markers. In combination with the previously demonstrated predictive value of live-imaging for differentiation potential<sup>[27]</sup>, we think that the measurement of cell motility is a fast assay which can be used alone or complementary to other tests in order to improve the prediction of clinical outcomes.

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## Abbreviations

MCS:	Mesenchymal stem cells
FBS:	Fetal bovine serum
PBMC:	Peripheral blood mononuclear cells
IL-2:	Interleukin-2
IL-4:	Interleukin-4
IL-6:	Interleukin-6
IL-10:	Interleukin-10
G-CSF:	Granulocyte-colony stimulating factor
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
MCP1:	Monocyte chemoattractant protein 1
TNF- $\alpha$ :	Tumor necrosis factor-alpha
GvHD:	Graft versus host disease

## Potential Conflicts of Interests

None

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